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14. ABSTRACT

Rational development of diagnostics and therapies for the transmissible spongiform encephalopathy (TSE) diseases requires a better understanding of the structure of the elemental unit of infectivity. Our laboratory is using a novel preparation of the TSE agent in a highly dispersed form to purify infectivity away from other molecular contaminants. Nuclease and protease enzyme treatments reduced the levels of some contaminants in the preparation without inactivating infectivity or causing aggregation. We have applied density gradient equilibrium ultracentrifugation to our preparation to separate particles based on the buoyant density of the infectivity. CsCl, a common density medium, proved unsuitable for our purposes because it caused aggregation of PrP^{res}. Despite this loss of dispersion, two sharp peaks of PrP^{res} and TSE infectivity were formed in the CsCl gradient. Subsequent studies with low ionic strength media have succeeded in concentrating PrP^{res} and purifying it away from greater than 99% of non-PrP protein while maintaining its small average particle size. The purified dispersed material is being titered by bioassay and the process is being scaled up. The purified particles are being carried forward to additional purification steps including equilibrium ultracentrifugation and sedimentation velocity ultracentrifugation.

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Introduction:

Purification and characterization of the infectious agent that causes transmissible spongiform encephalopathies would be a breakthrough in the development of diagnostic tests and treatments for the victims of these fatal neurological diseases. The monodisperse preparation (MP) produced in our laboratory from the brains of scrapie-infected hamsters is a novel starting material for the purification effort. Nuclease and protease treatments have resulted in a simplification of this complex mixture by lowering the concentrations and complexity of contaminating nucleic acids and proteins while preserving infectivity. Our early efforts to purify infectivity using density gradient ultracentrifugation revealed that the standard medium CsCl is not compatible with maintaining dispersion of PrP^{res}, the only known marker for scrapie infectivity. Now a three stage sucrose step gradient procedure has succeeded in purifying and concentrating PrP^{res} while maintaining its dispersed state. This indicates a likelihood that the scrapie agent has also been concentrated.

Scaled up production of purified scrapie infectivity involving liters of scrapie brain homogenate (SBH) is underway. We are actively pursuing the original plan of this project, which is to use equilibrium density, sedimentation rate, and other biochemical characteristics to concentrate, purify, and study the scrapie infectious agent. The techniques used are unbiased in their ability to isolate infectious particles, whether they are purely proteinaceous or contain other factors. Recently, a non-protein substance has been discovered co-purifying with PrP^{res}. This material is particulate and stains with silver nitrate, but is resistant to nucleases and proteases, even after denaturation. Its identification is proceeding.

Body:

The Aims referenced below are taken directly from the approved Statement of Work.

Aim I - To measure the sedimentation constant and the buoyant density of the infectivity in the monodisperse preparation and to compare these values to those of PrP^{res} in the preparation. The values obtained for the infectivity will be used to guide the purification procedures in Aim III.
CsCl Inactivation and Filtration Assay

CsCl is a standard medium used in the purification of viruses by equilibrium density gradient ultracentrifugation. Last year, we initiated an animal bioassay study to quantitatively measure any inactivation of scrapie infectivity caused by high concentrations of CsCl. The final results of this study have revealed that CsCl does cause an apparent two-thirds loss of infectivity after twenty-four hours incubation (See Table 1). This measurement is just barely statistically significant with the number of animals used in the endpoint dilution titration. Interestingly, NaCl had a greater effect, reducing apparent infectivity by approximately 87%. In contrast to monodisperse preparation (MP), unfiltered scrapie brain homogenate (SBH) did not lose any infectivity. Control groups showed that MP does not lose infectivity during incubation at room temperature after ten minutes or twenty-four hours. The explanation for the salt-induced loss of apparent infectivity does not seem to be osmotic shock, since the MP incubated with CsCl for ten minutes was unaffected and the infectivity in this sample saw the same rapid dilution of salt as the twenty-four hour group. As opposed to a true inactivation of the infectivity by CsCl and NaCl, we think it more likely that the infectious particles were caused to aggregate by the salt, reducing the number of independent particles.

CsCl causes aggregation of PrP^{res} particles in the MP and reduces apparent infectivity. During the last year, we identified the need for a rapid method to assess the dispersion state of PrP^{res} in our samples. The unique feature of our preparation is its highly dispersed character that may enable the isolation of elemental infectious particles different from the large PrP amyloid present in other SBH preparations. Treatments that cause a loss of this dispersion are contrary to our goals. While Asahi Planova nanofilters are the only filters with validated consistent pore sizes, there are other filters commercially available with small nominal pore sizes. The Whatman Anotop syringe filters were found to have low non-specific binding of PrP^{res} and were suitable for assessing the particle size distribution of PrP^{res}. Anotop filters are available in 0.2, 0.1, and 0.02 μm nominal pore sizes. We attempted to validate the Anotop 0.02 μm filter with the 27 nm bacteriophage PhiX174, but found that the filter failed to block the virus. Thus the pore sizes in the filter can not be uniformly 20 nm. As expected, all of the 35 nm Filtrate MP PrP signal passes through the 0.2 and 0.1 μm filters. 30-50% of the signal is blocked by the 0.02 μm filter, supporting the claim that the pore size is smaller than 35 nm. Upon incubation of MP with CsCl, a high degree of the PrP^{res} was blocked, not only by the 0.02 μm filter, but also by the 0.1 (See Figure 1). This Filtration Assay reveals that the average particle size of PrP^{res} increases considerably upon incubation in high concentrations of CsCl. As PrP^{res} is the only known marker for scrapie infectivity, it is highly likely that infectivity is also aggregating. Despite the fact that we sonicated our samples before dilution and inoculation in the endpoint dilution titration, some infectivity may have stayed clumped. Since a clump of infectious particles can only infect one animal whereas the unclumped particles would have infected more, the apparent titer is reduced.

We are pursuing mechanisms for counteracting the CsCl-induced aggregation and alternative density media, such as sucrose and Nycodenz, which will be discussed below.
CsCl Analytical Gradient

In a direct attempt to measure the equilibrium density of the infectious agent, we diluted MP

1:500 in phosphate buffered saline (PBS) without detergent and conducted equilibrium density gradient ultracentrifugation with a CsCl gradient. The high dilution before the gradient was intended to reduce the intermolecular associations of infectivity, including, theoretically, PrP to PrP aggregation. We observed last year that the presence of detergent in the CsCl gradient altered the equilibrium densities of both the PhiX174 and PCV viruses, lightening them. We wished to measure the intrinsic density of scrapie infectivity, so detergent was omitted. Before inoculating fractions from the gradient, the CsCl concentration had to be reduced to a level that was not toxic to hamsters. Instead of diluting the samples 1:100 in inoculation buffer (PBS with fetal calf serum), which would have lowered the level of infectivity below the limit of detection, we used gel filtration spin columns (Zeba brand from Pierce) to remove CsCl. Final results from this experiment show a drastic loss of infectivity wherever the spin columns were used (See Figure 2). We believe that CsCl caused aggregation of PrP^{res} and infectivity into large particles which were trapped among the beads of the spin column. What particles did make it through the columns and caused infections in the bioassay animals may not have been representative of the majority of infectious particles. Nevertheless, we report here that the area of the gradient with the most infectivity had a density range of 1.12 to 1.15 g/ml (fractions 26-32).

CsCl Preparative Gradient

An attempt was made to use CsCl density gradient ultracentrifugation to concentrate and purify large amounts of infectivity from undiluted monodisperse preparation (MP). MP was used as the diluent for CsCl powder to maximize the volume of infectious material included in the gradient. Following a twenty-four hour spin, fractions were collected, diluted 1:10 in inoculation buffer, sonicated, further diluted 1:30 and inoculated into hamsters for the incubation time bioassay. (No spin columns were used in this protocol; the fact that the sample was practically undiluted before the gradient allowed CsCl to be diluted out after the run.) Final results of this gradient showed two sharp peaks of infectivity that perfectly overlap with two peaks of PrP^{res} as defined by western blot (See Figure 3). Non-PrP protein was also concentrated into a broad peak in the same region of the gradient. Recovery of infectivity and PrP^{res} in the gradient were both 100%. In summary, infectivity and PrP^{res} were concentrated by the gradient into just six of 33 fractions, but purity was not increased relative to total protein and no separation of infectivity and PrP^{res} was achieved. The PrP^{res} particles in the peak were no longer dispersed so their properties were not those of an elemental form of infectivity, which is what we were seeking. The peak densities, 1.24 and 1.30 g/ml, may have been influenced by the aggregation of infectivity and possibly by the inclusion of other proteins non-specifically adhering to the aggregate. The fact that 100% of input infectivity was recovered shows that, if aggregates formed, they were dispersed by sonication prior to inoculation. This lack of any loss of infectivity is consistent with the theory that CsCl can aggregate scrapie infectivity but does not cause irreversible inactivation.

Sucrose and Nycodenz gradients

Due in part to the support of our NPRP grant, we were able to purchase an SW32 rotor for ultracentrifugation. This is the highest volume swinging bucket rotor available and is capable of generating higher g forces than the SW28 in addition to some design improvements. Our work with sucrose and Nycodenz gradients has utilized the advantages of the SW32.

Equilibrium density gradient ultracentrifugation is a technique used to concentrate particles at a characteristic density without impacting them against a hard surface, such as the bottom of a centrifuge tube. Long centrifugation runs at high speed have revealed that PrP^{res} has a density in sucrose greater than 1.32 g/ml, the density of a 65% sucrose solution. Thus a higher concentration of sucrose would be needed to match the density of PrP^{res} and prevent it from collecting at the bottom of the tube. Due to the viscosity of such sucrose solutions (hundreds to thousands of times that of water), sucrose is not suitable for equilibrium runs. I will explain under Aim III how sucrose

has been used in a short step gradient procedure.

Nycodenz is one of the iodothalamic acid derivatives used as alternative density media. Nycodenz solutions are much less viscous than sucrose solutions. In preliminary experiments, it has proved compatible with equilibrium ultracentrifugation. The density of PrP^{res} in Nycodenz is roughly 1.10 to 1.20 g/ml, or less than the density of a 60% solution. Importantly, Nycodenz does not cause aggregation of PrP^{res} the way that CsCl does. Current plans call for developing conditions for a continuous gradient of Nycodenz, looking first at the behavior of PrP^{res}, then adding the control viruses PhiX174 and PCV. The control viruses will validate that the gradient is capable of concentrating particles of viral size. Finally, we will inoculate fractions from a final gradient to assay for scrapie infectivity. The gradient will be assessed for its ability to concentrate infectivity as well as separate it from contaminants.

Sedimentation Velocity

One of the tasks in our statement of work is the measurement of the sedimentation rates of PrP^{res} and infectivity from the MP. The final experiment will involve a short (one to eight hour) spin in an ultracentrifuge of MP layered over a 5-20% sucrose gradient. Larger and denser particles sediment faster through the sucrose gradient so that heterogeneous particles reach different locations in the centrifuge tube. In this type of "velocity run", particles are not expected to reach their equilibrium density and stop migrating, so the high density of PrP^{res} in sucrose will not be a problem. The run time will be optimized to end before any particles reach the bottom of the tube. This protocol will also be developed by assaying fractions for PrP^{res} first, then PhiX174 and PCV, and then a perfect run will be inoculated for animal bioassay of scrapie infectivity. Preliminary experiments using differential centrifugation and varying run times and speeds indicated that the average sedimentation constant of PrP^{res} in MP is approximately 120 Svedbergs with a wide range of smaller and larger particles.

Aim II. To obtain a monodisperse preparation of TSE infectivity from 10% brain homogenate instead of 1% homogenate, thereby increasing the infectivity of the preparation by a factor of ten.

A matrix of SBH and detergent concentrations were tested for clarity and filterability. Every combination produced a clear solution after 0.2 μm filtration, but upon freezing and thawing, many again became cloudy. The ratio of detergent to homogenate and their absolute concentrations were important in creating clarified solutions. It is our current understanding that turbidity in these solutions is caused by lipid aggregates which can be dispersed with detergent and extrusion through the pores of a filter. Insufficient detergent allows lipid aggregates to reform, a process that in enhanced at low temperature. It was found that 10% SBH could be clarified with 10% detergent, however this solution was difficult to filter. The mixture of 4% SBH and 2% detergent was easier to use and potentially increased infectivity over the original MP by a factor of four.

We have obtained an agreement with Asahi Corporation to provide us with additional nanofilters at no charge and will be using them to produce new MP from 4% SBH. In the meantime, we have used alternative filtration to generate a dispersed suspension of SBH. After screening eight different membranes for binding of PrP^{res}, the polyethersulfone (PES) membrane material was chosen for filtration of SBH. Pall Corporation's Supor PES membrane is available in various sizes for filtration of 1 ml to 5 L. As projected in the Statement of Work, a small lot (50 ml) was prepared and tested. 4% SBH was mechanically and chemically dispersed and then digested with Benzonase Nuclease and the protease trypsin, thus incorporating those formerly separate treatments into the protocol for making MP. The treated homogenate was then put through an Acropak brand filtration capsule with a 0.8 μm pre-filter and a 0.2 μm final filter. This lot was characterized and applied to the sucrose step gradient procedure described elsewhere in this report.

The PrP^{res} in the Acropak Filtrate behaved similarly to the original MP, so a two liter lot was prepared. This new starting material will be used in purification experiments in addition to Asahi filtrates. Animal bioassays have been initiated to define the titer of the 4% scrapie brain preparation before and after the Acropak filter.

Characterization of the filtrate from the Acropak has shown that recovery of PrP^{res} is near 100%. According to quantitative western blot, which uses endpoint dilution of samples to compare equivalent signals, the Acropak Filtrate has 100 times the PrP^{res} as the 35 nm Filtrate MP (See Figure 4). The Filtration Assay, developed during the last year was used to assess the dispersion state of the PrP^{res} particles in the Acropak Filtrate and it was found that a high percentage of the PrP signal was able to pass through the Anotop 0.02 μm filters, indicating a small average particle size, even without nanofiltration through an Asahi filter.

Aim III. To purify the monodisperse infectivity using sedimentation to equilibrium, sedimentation velocity, and other methods as necessary including column chromatography and electrophoresis.

Treatments

Two endpoint dilution titrations have proved that the scrapie infectivity in MP is unaffected by storage at -80°C for four years or digestions with Benzonase Nuclease and the protease trypsin. This second treatment with enzymes greatly reduces the complexity of the preparation by removing free nucleic acids and trypsin-sensitive proteins. According to the Filtration Assay, PrP^{res} maintains its dispersed state after the digestions. Benzonase and trypsin treatments are now standard parts of the procedure for making MP.

Tryptic Digestion Assay

After optimization of the tryptic digestion of MP, the knowledge gained was applied to the development of a screening assay, the Tryptic Digestion Assay, that identifies brain homogenates positive for PrP^{res}. This assay has advantages over the commonly used digestion with Proteinase K. PrP^{res} is only partially resistant to the aggressive protease Proteinase K, which is able to digest PrP^{res} if a high enough concentration of enzyme is applied or if the reaction takes place at a high temperature. Trypsin, however, is incapable of digesting PrP^{res} even at elevated temperature or when 5 mg/ml enzyme are added (50-fold the necessary concentration). A coded study, internal to our lab, was conducted using mouse brain homogenate from normal mice and mice infected with bovine spongiform encephalopathy (BSE) and the assay correctly identified the three positive samples and the seven negatives (See Figure 5).

Sucrose Step Gradient

A step gradient ultracentrifugation procedure uses layers of defined density to separate components of a mixture. In our gradients, PrP^{res} particles sediment from the sample zone, which has no sucrose and fills the top two-thirds of the centrifuge tube, through the intermediate sucrose zone, and slow their progression at the shelf between the intermediate zone and the dense sucrose cushion. Particles move through the intermediate zone because of their high sedimentation rate while soluble proteins and other contaminants remain in the sample zone or only slightly penetrate the intermediate layer. The sucrose cushion slows the migration of PrP^{res} particles because it has a density closer to that of PrP^{res} and its viscosity is many times that of water. The Sucrose Step Gradient ultracentrifugation procedure was performed using 200 ml of MP and fractions were collected from the bottom of the centrifuge tube. The fractions containing the concentrated PrP^{res} were loaded onto a second step gradient and spun again. After a third run, PrP^{res} was found to be concentrated 30-fold while non-PrP protein was reduced by 99% (See Figures 6 and 7). The bioassay for infectivity has been initiated.

Silver staining contaminant

Coomassie stain revealed no contaminants among the step gradient's purified particles, so we used silver stain, a more sensitive technique, to determine the level of residual non-PrP protein (See Figure 7). No protein bands were visible, but a surprisingly strong stain was seen near the top of each sample lane. This material, which is resistant to nucleases and proteases, appears to be unknown in TSE literature. The unidentified substance seems to co-purify with PrP^{res} particles because it becomes more concentrated with each successive run of the step gradients. We are considering the possibility that some of the contaminant particles are associated with PrP^{res} particles and may play a role in the sedimentation of PrP^{res} and perhaps even in pathogenesis. Many properties of this "silver staining contaminant" have been characterized. When electrophoresed in a gradient gel, a smear of staining is seen, indicating a heterogeneous mixture of molecular weights (See Figure 8). Current plans include using acid and alkaline hydrolysis and mass spectroscopy to identify the subunits of the contaminant.

PH Study

The infectious agent of the transmissible spongiform encephalopathies has been notoriously difficult to purify in a dispersed form and success in such a purification effort may depend on using every available advantage. Many viral purification procedures are limited to gentle conditions that do not inactivate the virions, but the scrapie agent is unconventional among infectious agents in its resistance to extremes of heat and pH. The possibility exists that an elevated pH may improve the efficiency of a future purification step, such as a density gradient or column chromatography, and allow scrapie infectivity to be concentrated in a dispersed form. A large scale bioassay study of the stability of the scrapie agent to increasing alkalinity has been initiated. Early indications are that infectivity is stable at pH 9, reduced at pH 10, and eliminated at pH 11. These results may guide the adjustment of pH during upcoming protocols.

Substrate	Treatment	Exposure	$Log_{10}ID_{50}$ /g brain	ID_{50} /g brain
Monodisperse Preparation	PBS	10 min	6.80	$6.32x10^6$
Monodisperse Preparation	PBS	24 hrs	6.80	$6.32x10^6$
Monodisperse Preparation	CsCl	10 min	6.80	$6.32x10^6$
Monodisperse Preparation	CsCl	24 hrs	6.30	$2.00x10^6$
Monodisperse Preparation	NaCl	24 hrs	5.93	$8.43x10^5$
Scrapie brain homogenate	CsCl	24 hrs	9.97	$9.28x10^9$

Table 1. Incubation of scrapie infectivity with a high concentration of CsCl for twenty-four hours led to no loss of infectivity from scrapie brain homogenate and a small loss from monodisperse preparation. Incubation with 2.5 M NaCl led to a more substantial loss of infectivity, 87%. The control incubation of monodisperse preparation in the same buffer it was made in, PBS with detergent, demonstrated the stability of infectivity. The preservation of infectivity after a ten minute incubation in CsCl confirms that osmotic shock from rapid dilution is not the cause of lost infectivity. High salt concentrations most likely do not cause true inactivation of scrapie infectivity, but can generate an apparent loss of titer.

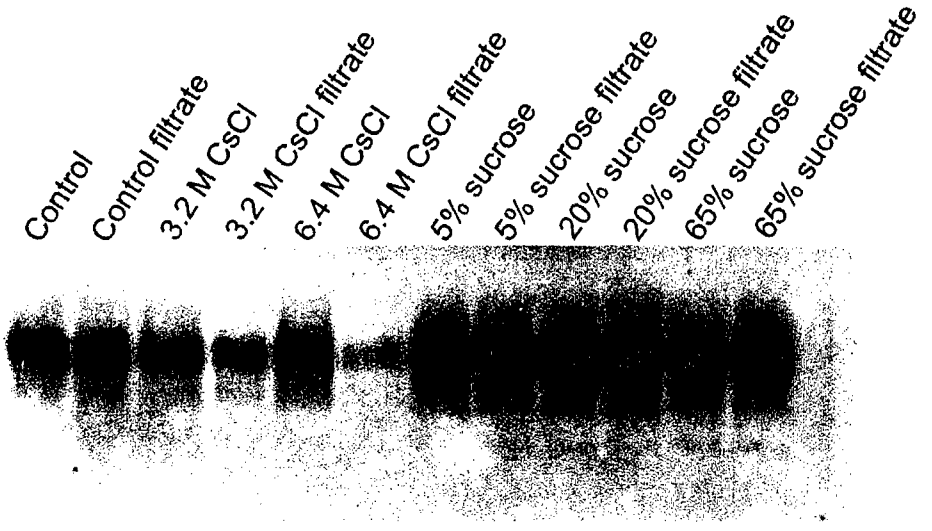

Figure 1. A high concentration of CsCl induces aggregation of PrP^{res} from the monodisperse preparation. Following incubation with CsCl, PrP^{res} that previously passed through a 35 nm pore size nanofilter was unable to penetrate a 220 nm filter. Sucrose did not cause a loss to the filter. The average particle size of PrP^{res} increases upon incubation in CsCl.

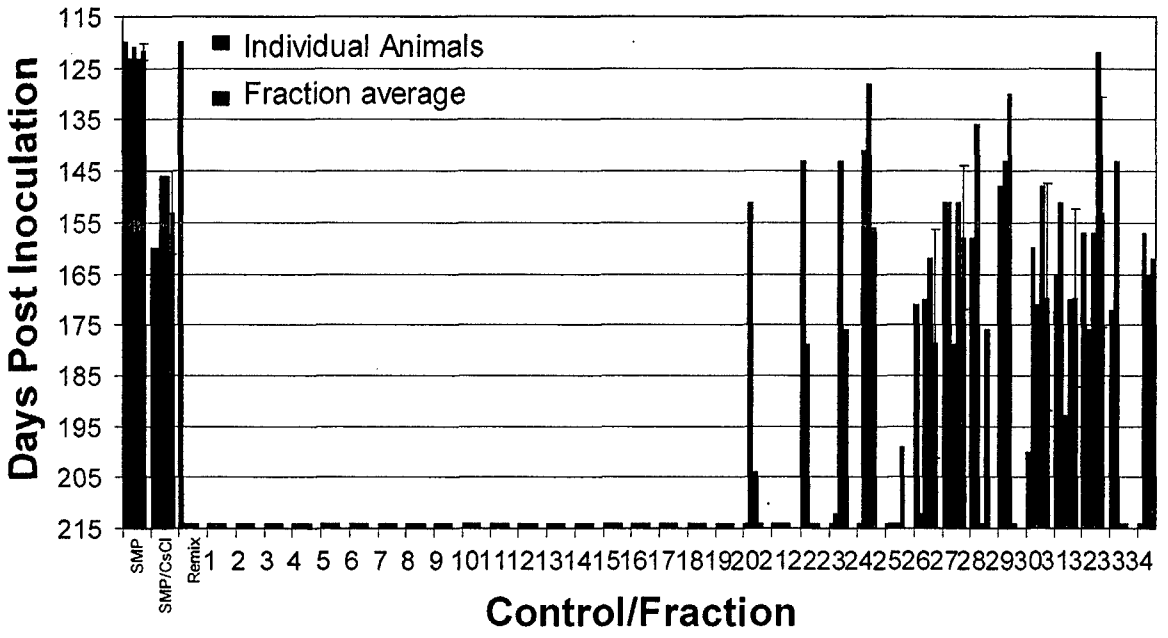

Figure 2. Recovery of infectivity from the Analytical CsCl Gradient was poor. Control monodisperse preparation caused illness in hamsters with an incubation time of 123 days. Monodisperse preparation incubated with CsCl and then passed through spin columns to reduce the CsCl concentration to a level non-toxic to hamsters caused illness in 153 days. The increase in incubation time indicates a loss of infectivity. Fractions from the gradient were also passed through spin columns and very little infectivity was recovered from any point in the centrifuge tube. It is most likely that, in the presence of CsCl and the absence of detergent, PrP^{res} and infectivity aggregated and were trapped in the spin column resin and thus were not inoculated.

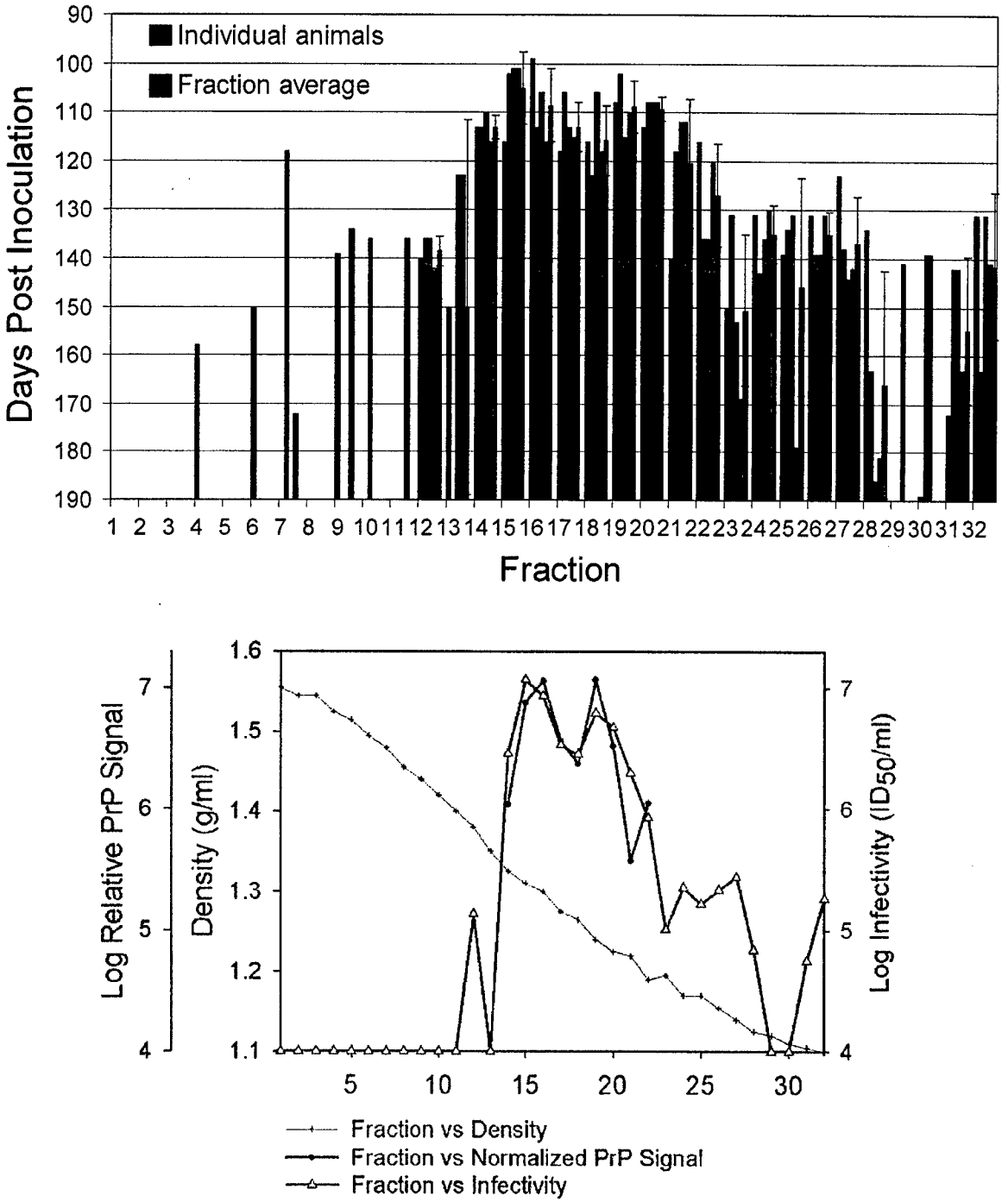

Figure 3. In the Preparative CsCl Gradient, scrapie infectivity and PrP^{res} were concentrated into the same two peaks covering one-sixth of the total gradient. The peak densities were 1.24 and 1.30 g/ml, but in light of the Cs Inactivation, CsCl Analytical Gradient, and Filtration Assay results, it is likely that these densities are not characteristic of elemental particles. Additionally, non-PrP proteins were concentrated into a broad peak in the same area of the gradient, so no purification was achieved despite the focusing of PrP^{res} and infectivity.

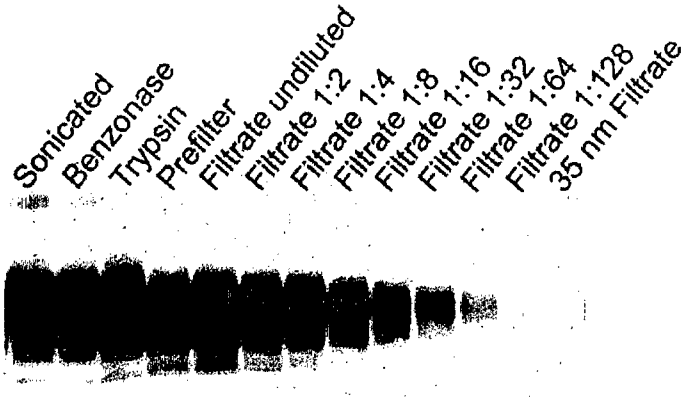

Figure 4. The dispersed PrP^{res} in the two liters of Acropak Filtrate is at approximately 100-fold the concentration of PrP^{res} in the 35 nm Filtrate monodisperse preparation. A 1:128 dilution of Acropak Filtrate had a western blot signal greater than that of a 1:2 dilution of 35 nm Filtrate monodisperse preparation. Quantiation of these signals allows calculation of the ratio of PrP^{res} content in the Acropak Filtrate and 35 nm Filtrate. This difference is due to the use of 4% scrapie brain instead of 1%, the larger pore size of the Acropak filter (200 nm vs 35 nm), and the lack of non-specific adsorption to the Acropak membrane. Substantial loss of PrP^{res} and infectivity is expected when this material is passed through an Asahi nanofilter. The Acropak Filtrate will be useful as a stock starting material for many experiments due to its high content of the infectivity marker, PrP^{res}.

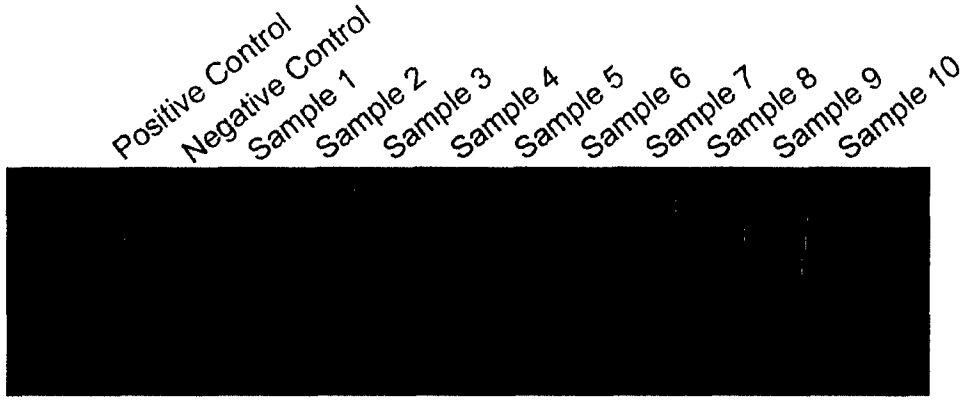

Figure 5. A coded study of the Tryptic Digestion Assay was conducted in our lab and the assay correctly identified three positive samples and seven negative samples. Unlike normal mouse brains, the brains of mice infected with bovine spongiform encephalopathy contain PrP that is resistant to proteolytic digestion and can be detected by western blot. After being informed of difficulties with the standard Proteinase K method, I developed this assay that uses a high concentration of trypsin to remove PrP^{C} while leaving PrP^{res} intact. With PrP^{C} completely removed, there are no false positives and with PrP^{res} unaffected, false negatives are avoided. All results with the assay have so far been unambiguous and reproducible enabling rapid determination of the infection status of an animal.

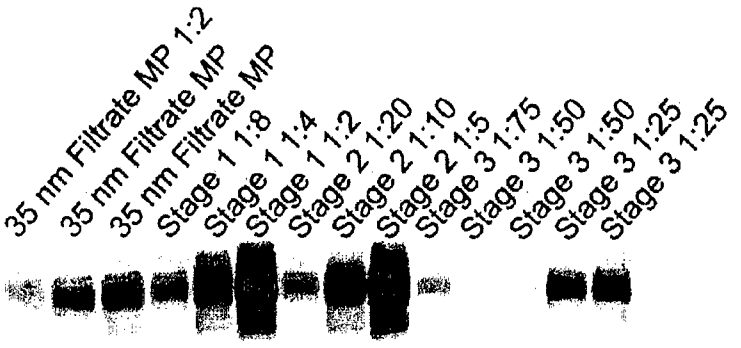

Figure 6. Sequential sucrose step gradients were used to concentrate PrP^{res} 30-fold from 35 nm Filtrate monodisperse preparation. Quantitative western blot was used to demonstrate that a 30-fold dilution of the final purified particles gives a signal equal to undiluted starting material. The concentration factors after the first two stages of the step gradient procedure were 5-fold and 12-fold.

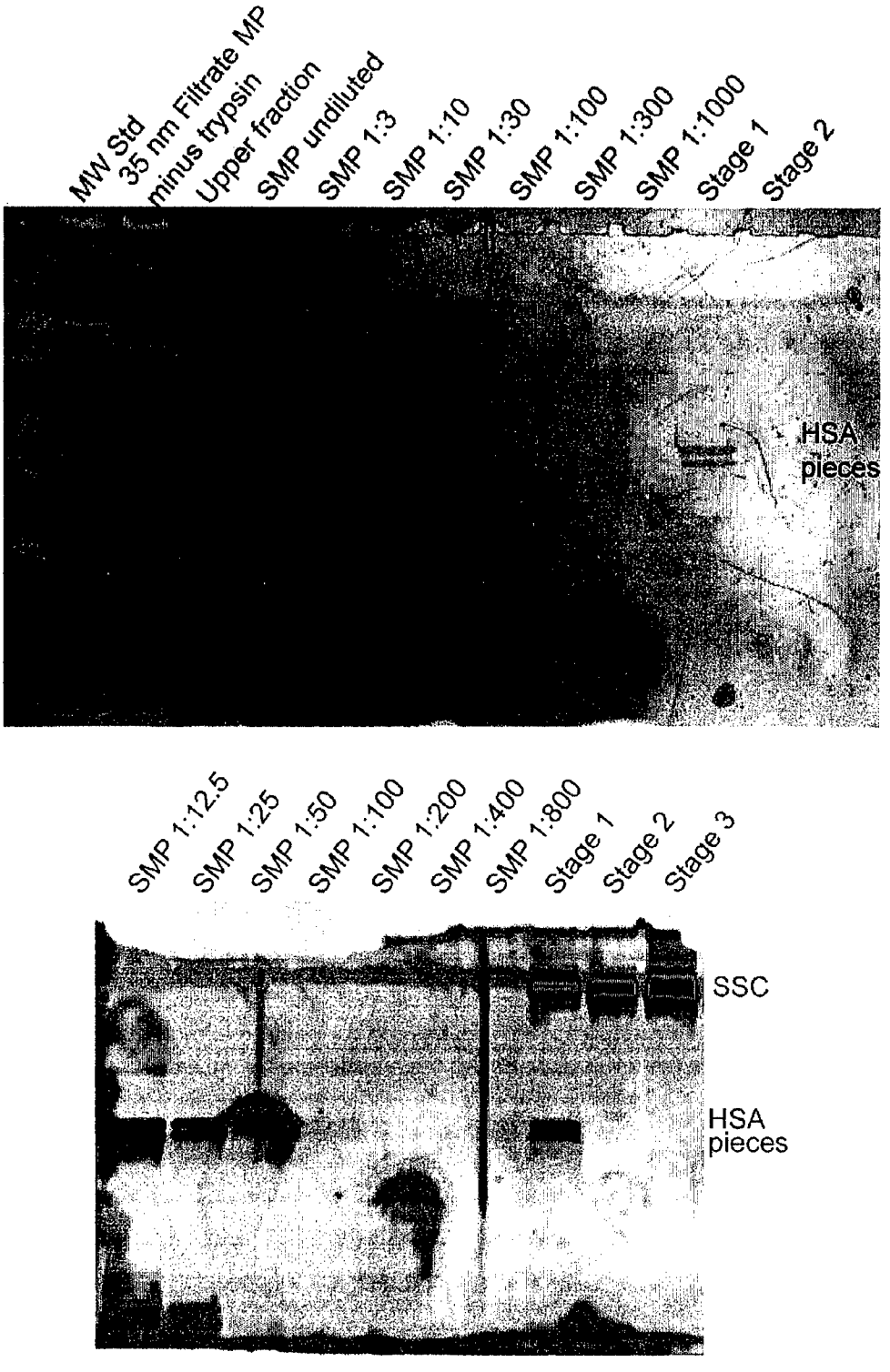

Figure 7. (See next page)

Figure 7. (Previous page) The purified PrP^{res} particles from the second and third stages of the step gradient procedure have less than 1% of the non-PrP protein as the starting material, monodisperse preparation (MP) digested (simplified) with Benzonase and trypsin (SMP). Dilution of the digested MP and staining with silver in the bottom image allows us to see a faint band of trypsin-resistant human serum albumen (HSA) pieces at a 1:100 dilution. Since the stage two and stage three pooled fractions have no detectible protein staining, we can conclude that they contain less than $1/100^{th}$ of the protein of the starting material. The stage one material, by contrast, clearly still contains protein contamination, visible even by coomassie blue stain in the top image.

The silver stained bottom image reveals the silver staining contaminant, a substance that migrates like a high molecular weight protein, mostly moving through the 4% stacking gel and then migrating slowly in the 15% resolving gel. This material, which does not appear to be protein is not stained by coomassie blue, as seen in the top image.

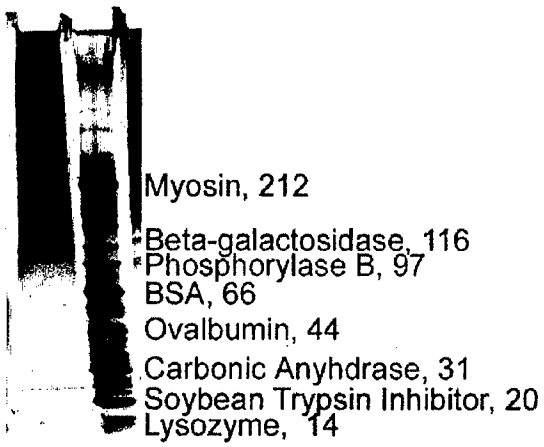

Figure 8. The silver staining contaminant (SSC) appears as a smear of heterogeneous species in SDS-PAGE with a 4-15% gradient gel and silver stain. Although the material enters the gel and runs in the same direction as protein, it does not seem to be protein because it is completely resistant to Proteinase K (even after denaturation) and does not stain with coomassie blue or other protein-specific stains. The MW standards in the lane next to the SSC might give the appearance that the contaminant has an average molecular weight higher than myosin (212 kDa), but without knowing the properties of the material, the comparison is meaningless. Migration in SDS-PAGE is affected by charge, binding to SDS, and the shape of a macromolecule in addition to molecular weight. It is clear from this image, however, that the SSC is a mixture of species with different migration rates in SDS-PAGE.

Key Research Accomplishments:

The Aims referenced below are taken directly from the approved Statement of Work.

Aim I - To measure the sedimentation constant and the buoyant density of the infectivity in the monodisperse preparation and to compare these values to those of PrP^{res} in the preparation. The values obtained for the infectivity will be used to guide the purification procedures in Aim III.

- Animal bioassay study completed that demonstrated a small loss of scrapie infectivity following incubation with a high concentration of CsCl or NaCl.
- The Filtration Assay was developed to quickly assess the aggregation state of PrP^{res}. This assay revealed that CsCl induces aggregation of PrP^{res}, a change that may cause the appearance of a loss of infectivity.
- Bioassay of the CsCl Analytical Gradient completed. Recovery of infectivity was very low in this experiment, probably due to aggregation of infectivity and subsequent capture by the gel filtration spin columns used in the procedure.
- CsCl Preparative Gradient bioassay completed. Infectivity was found concentrated in two peaks at densities of 1.24 and 1.30 g/ml. Non-PrP proteins and PrP^{res} were also restricted to the same fractions, thus concentration was achieved, but not purification.
- Sucrose has been determined to be unsuitable for equilibrium density gradient ultracentrifugation, but Nycodenz is a compatible medium. The density of PrP^{res} from Monodisperse Preparation (MP), in sucrose, is greater than that of a 65% sucrose solution (1.32 g/ml). In Nycodenz, it is roughly 1.10 to 1.20 g/ml, or less than the density of a 60% solution.
- The average sedimentation constant of PrP^{res} particles in the MP has been determined to be approximately 120 Svedbergs using differential centrifugation with varying run times and speeds.

Aim II. To obtain a monodisperse preparation of TSE infectivity from 10% brain homogenate instead of 1% homogenate, thereby increasing the infectivity of the preparation by a factor of ten.

- A matrix of homogenate and detergent concentrations were tested for clarity and filterability.
- A test lot of 50 ml of 4% scrapie brain homogenate (SBH) was filtered using a polyethersulfone (PES) membrane. This filtrate was characterized, including application of the sucrose step gradient procedure.
- A production lot of two liters of 4% SBH PES Filtrate was produced and characterized.

Aim III. To purify the monodisperse infectivity using sedimentation to equilibrium, sedimentation velocity, and other methods as necessary including column chromatography and electrophoresis.

- Endpoint dilution titration in the animal bioassay has proved that MP retains its infectivity after four years at -80°C.
- Following simplification of the MP by digestion with Benzonase Nuclease and the protease trypsin, another titration verified that no infectivity had been lost.
- After optimization of the tryptic digestion of MP, the knowledge gained was applied to the development of a screening assay, the Tryptic Digestion Assay, that identifies brain homogenates positive for PrP^{res}. This assay has advantages over the commonly used digestion with Proteinase K.

- The Sucrose Step Gradient ultracentrifugation procedure was performed using MP and PrP^{res} was concentrated 30-fold while non-PrP protein was reduced by 99%. The bioassay for infectivity has been initiated.
- Nycodenz step gradients have also been proven capable of concentrating PrP^{res} while separating it from other proteins.
- An unidentified substance has been found that co-purifies with PrP^{res} particles in density step gradients. This material, which is resistant to nucleases and proteases, appears to be unknown in TSE literature. Many properties of this "silver staining contaminant" have been characterized.
- A large scale bioassay study of the stability of the scrapie agent to increasing alkalinity has been initiated. Early indications are that infectivity is stable at pH 9, reduced at pH 10, and eliminated at pH 11. These results may guide the adjustment of pH during future purification steps.

Conclusions:

We have established, through a one-year animal bioassay, that the scrapie infectivity in our monodisperse preparation (MP) is stable to incubation at -80°C and digestion with Benzonase Nuclease and the protease trypsin. Although CsCl was used to measure peak densities for scrapie infectious particles and succeeded in concentrating infectivity from our monodisperse preparation, this density medium has been deemed unsuitable for work with the scrapie agent because it causes aggregation of PrP^{res}, the only known marker for scrapie infectivity. The density media sucrose and Nycodenz do not cause this aggregation and therefore may help us to achieve our goal of measuring the density of the most elemental form of the agent free from interactions with other scrapie particles or other molecules. Sucrose will be used in a velocity sedimentation procedure to measure the sedimentation constant of infectivity while separating it from contaminants. Sucrose has also been used in a step gradient procedure to concentrate PrP^{res} 30-fold while removing 99% of non-PrP protein. A Nycodenz continuous density gradient will be used to separate scrapie infectivity from contaminants based on the particles' equilibrium densities, which will also be defined during the experiment. An unidentified substance that co-purifies with PrP^{res} particles in density step gradients is being investigated for its relationship to PrP^{res} and effect on infectivity.